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Specific Monoclonal Antibody-Based Enzyme Immunoassay for Sensitive and Reliable Detection of Alternaria Mycotoxin Iso-Tenuazonic Acid in Food Products

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1 **Specific monoclonal antibody based enzyme immunoassay for**
2 **sensitive and reliable detection of Alternaria Mycotoxin**
3 **Iso-Tenuazonic Acid in food products**

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21 **ABSTRACT:** In this paper, we report the development of a sensitive and specific
22 monoclonal antibody based immunodiagnostic method for the detection of
23 iso-tenuazonic acid (ITeA), an *Alternaria* mycotoxin, in food samples. The ITeA was
24 derivatized with hydrazine hydrate to produce the antigen
25 (E)-3-(1-hydrazonoethyl)-4-hydroxy-5-isobutyl-1H-pyrrol-2(5H)-one (ITeAH) which
26 was further reacted with glyoxalic acid to generate the hapten
27 (E)-2-((Z)-(1-(4-hydroxy-5-isobutyl-2-oxo-2,5-dihydro-1H-pyrrol-3-yl)ethylidene)
28 (ITeAHGA) which was used as an immunogen after conjugation to bovine serum
29 albumin (BSA). A highly specific monoclonal antibody selectively binding to ITeAH
30 was generated via the hybridoma technique and subsequently used to construct a
31 heterologous indirect competitive enzyme-linked immunosorbent assay (icELISA)
32 using ITeAH as the competitive antigen for the detection of ITeA with a limit of
33 detection (LOD) of 0.5 ng/mL. Under the optimum conditions, the developed
34 icELISA is highly sensitive (IC_{50} = 7.8 ng/ml) with recovery rates ranged from 82.3 to
35 109.8% for spiked food samples. The comparative analysis of results revealed a good
36 correlation between the icELISA and the standard HPLC-MS/MS method, confirming
37 the suitability of the developed icELISA for screening and detection of mycotoxin
38 ITeA in food samples.

39 **KEYWORDS:** *iso-tenuazonic acid; monoclonal antibody; enzyme-linked*
40 *immunosorbent assay; mycotoxin*

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43 **1. Introduction**

44 Tenuazonic acid (TeA) and its isomer, iso-tenuazonic acid (ITeA), are major
45 metabolic toxic products of *Alternaria* and other fungal species such as *Aspergillus*
46 *flavus*, *Pyricularia oryzae*, and *Phoma sorghina* (Qiang et al. 2008; Marin et al. 2013).
47 Owing to their ability of growth at low temperature, *Alternaria* species are responsible
48 for spoilage of food plants during refrigerated transport and storage, whilst some
49 *Alternaria* mycotoxins are heat-resistant even at relatively high temperature of 230°C
50 and thus can not be detoxicated by cooking (Siegel et al. 2010). TeA-producing fungi
51 are ubiquitous in many biological environments and capable of infecting most plant
52 species including food crops. In fruits and vegetables, TeA has the highest
53 contamination frequency and is present in higher concentrations compared to other
54 *Alternaria* toxins (EFSA 2011). In spite of being cautious pathogens of many plant
55 diseases, genotoxic and fetotoxic in rats, as well as being linked to the development of
56 oesophageal cancer, currently there are no regulations on *Alternaria* toxins in food
57 and feed in Europe or in other regions of the world. Furthermore, TeA is considered
58 the most acutely toxic *Alternaria* mycotoxins (Shephard et al. 2012). Because of the
59 similarities in chemical structure, it is speculated that ITeA and TeA are of similar
60 toxicological relevance. For instance, they both exhibit remarkable toxic effects on
61 *Artemia salina* with mortality rates of 68.9% and 73.6%, respectively (Qin et al. 2009).
62 Not only the antibacterial activity of ITeA is identical to TeA (Gitterman 1965), ITeA
63 also exhibits significant phytotoxicity inhibiting plant growth and promoting leaf
64 browning (Lebrun et al. 1988).

65 It was reported that some naturally contaminated food commodities contained only
66 4% ITeA in their total TeA content (Asam et al. 2013), but the high level of ITeA in

sorghum based infant food has raised increasing concerns and more samples should be analyzed to elucidate if there is a general tendency related to sorghum (Qiang et al. 2008). Nevertheless, TeA has been found to be the predominant *Alternaria* mycotoxin detected in China in all tomato ketchup (10.2-1781 µg/kg) and tomato juice samples (7.4-278 µg/kg) and in 99.4% of wheat flour (1.76-520 µg/kg) (Zhao et al. 2015a,b). Therefore, the total exposure of ITeA can not be neglected due to its acute toxicity and potential harmful effects on human and animal health.

Subsequently, it is necessary to continually monitor ITeA and TeA in fruits, vegetables, cereals and oleaginous plants intended for human consumption and feed production (Qiang et al. 2008). Although several instrumental methods exist for measuring of TeA and its analogues (Noser et al. 2011; Siegel et al. 2009; Asam et al. 2011; Prella et al. 2013), LC-MS is the only instrumental technique available for ITeA (Asam et al. 2013). The method simultaneously detects both TeA and ITeA after their derivatization with 2,4-dinitrophenylhydrazine. While instrumental methods can offer a high level of precision and accuracy, the sophistication aspect of such analytical tools rendering their limited applications in routine and high throughput analysis. Immunochemical methods, on the other hand, are simple and cost effective, yet sensitive and rapid, enabling for a large array of sample screening. Immunoassay for TeA has recently been described in a couple of studies (Gross et al. 2011; Yang et al. 2012). Production of an antibody to the analyte is essential to an immunoassay. Compared with polyclonal antibody which is widely used in immunoassay, monoclonal antibody is more specific and homogenous, also more difficult to produce. There is no report on ITeA immunoassay based on monoclonal antibody available to date to the best of our knowledge. The present study therefore aimed to develop a sensitive and specific immunochemical screening method and monitor ITeA in food

products.

In the present study, two novel ITeA haptens, ITeAH and ITeAHGA (Fig.1), were adopted to develop a specific anti-ITeAH antibody, using ITeAHGA as a hapten to prepare the immunogen by coupling to a carrier protein (BSA). A highly specific monoclonal antibody (mAb) selectively binding to ITeAH, was generated via the hybridoma technique and was subsequently used to develop an icELISA for the detection of ITeA. Various ELISA conditions were optimized and performance of the assay was evaluated by measuring ITeA in real food samples.

2. Materials and methods

2.1 Reagents and Chemicals

Leucine, bovine serum albumin (BSA), dicyclohexylcarbodiimide(DCC), N-hydroxysuccinimide(NHS), polyethylene glycol (PEG) 2000, ovalbumin (OVA), 3,3',5,5'-tetramethylbenzidine(TMB), complete and incomplete Freund's adjuvants, hypoxanthine-aminopterin-thymidine (HAT), hypoxanthine-thymidine (HT), culture media RPMI-1640, and pristane were obtained from Sigma (St. Louis, MO, USA). The mouse SP2/0 myeloma cell line was sourced from the Sun Yat-sen University (Guangzhou, China). Tween-20, *N,N*-Dimethylformamide (DMF), sodium ethylate, diketene, benzene, ethyl acetate, chloroform, hydrazine hydrate, methanol, glutaric dialdehyde, and 4-hydroxybenzaldehyde were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Horseradish peroxidase-labeled goat anti-mouse IgG (IgG-HRP) was obtained from Boster Biotech Co., Ltd. (Wuhan, China). Polystyrene microtiter plates were sourced from Jiete Biotech Co., Ltd. (Guangzhou, China). Microwell plates for cell culture were obtained from Corning Incorporated (New York, USA). All organic solvents and chemicals used were of analytical grade. Female Balb/c mice were purchased from Guangdong Medical

Laboratory Animal Center. The mycotoxin standards of AOH, AME were purchased from Taileqi Technology Co., Ltd. (Beijing, China) and TeA, ITeA, ITeAH were synthesized in Guangdong Provincial Key Laboratory of Food Quality and Safety (Guangzhou, China).

2.2 Buffers and Solutions

Buffers were prepared and used as follows: 10 mmol/L PBST (PBS buffer containing 0.1% Tween-20) for washing; 50 mmol/L carbonate buffer (pH 9.6) for coating, 5% of skimmed milk powder in PBS buffer for blocking, sodium phosphate buffers (pH 5.4) as general diluent, and 2 mol/L H₂SO₄ was used as the stop solution. Chromogenic reagent was prepared using 150 µL of the TMB solution (15 mg/mL in DMF) and 2.5 µL of 6% (w/v) H₂O₂ in 10 mL of 0.1 mol/L citrate.

2.3 Instruments

Centrifuge (5810R) was purchased from Eppendorf Company, USA. The LC-MS/MS analysis was carried out using a 1,200 series LC system (Agilent, USA) equipped with the Agilent 6410 Triple Quad LC-MS System. The analytical column was 2.1 mm×150 mm, 3.5 µm Zorbax SB-C18. Nuclear magnetic resonance (NMR) spectra were obtained with DRX-600 NMR spectrometers (Bruker, Germany-Switzerland). Ultraviolet-visible (UV-vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan). Microtiter plates were washed using a Multiskan MK2 microplate washer (Thermo Labsystems, USA). The Optical Density (OD) of ELISA signals were measured using a Perkin Elmer 1420 Multi-label Analyzer (USA). Wrist-action shaker (Vortex Genius3) was a product of IKA Company, Germany.

2.4 Synthesis and Characterization of Haptens

2.4.1 ITeA synthesis

ITeA was synthesized according to the method previously described (Yang et al. 2012) (Fig. 2). After recrystallization in chloroform, a white needle solid was obtained with a 38.6% yield. The ITeA structure was confirmed by APCI-MS and NMR analysis. Two haptens namely, ITeAH ((*E*)-3-(1-hydrazonoethyl)-4-hydroxy-5-isobutyl-1*H*-pyrrol-2(5*H*)-one) and ITeAHGA ((*E*)-2-((*Z*)-(1-(4-hydroxy-5-isobutyl-2-oxo-2,5-dihydro-1*H*-pyrrol-3-yl)ethylidene)hydrazono)acetic acid) were subsequently synthesized following the procedures shown in Fig. 2.

2.4.2 ITeAH synthesis

The synthesis was carried out by the Wolff-Kishner reaction. Briefly, ITeA (1.85 g, 10 mmol) was dissolved in 20 mL chloroform and added dropwise into the flask containing 20 mL of hydrous hydrazine hydrate. After mixing for 1h, 20 mL distilled water was added, and the mixture was then extracted twice with chloroform. The organic phase was then washed with water and dried over anhydrous magnesium sulfate. The solvent was removed to obtain a gray solid of ITeAH with a 65% yield.

2.4.3 ITeAHGA synthesis

The mixture of ITeAH (1.99 g, 10 mmol) and 2-oxoacetic acid (0.89 g, 12 mmol) was dissolved in 20 mL chloroform and agitating for 2h to produce a white solid of ITeAHGA, with a 48% yield.

2.5 Preparation and characterization of hapten-protein conjugates

The ITeAH hapten was conjugated to OVA via the glutaraldehyde method (Hamajima et al. 1995) in the following procedures and used as a coating antigen: OVA (1.66 μ mol/L) and ITeAH (166 μ mol/L) were first prepared in PBS (pH7.4), and 60 μ L of glutaric dialdehyde was then added dropwise. The mixture was gently stirred for 12h at 4°C and purified by dialyzes against PBS (10 mmol/L, pH 8.0) for two days.

The dialyzed product was centrifuged for 10 min and the supernatant was collected and stored at 4°C. The structures of the final conjugates were confirmed by a UV-vis (200-500 nm) spectroscopy.

The ITeAHGA hapten was conjugated to BSA and OVA via the active ester method (McAdam et al. 1992) to prepare the immunogen and coating antigen, respectively. Briefly, ITeAHGA (0.166 μ mol), DCC (0.122 μ mol), and NHS (0.122 μ mol) were dissolved in 1.0 mL of DMF and the mixture was gently stirred at 4°C overnight. After centrifugation for 10 min, 500 μ L of the supernatant was collected and added dropwise to 10 mL of PBS (10 mmol/L, pH 8.0) containing BSA or OVA (with molar ratio of carrier protein to antigen at 1:60). The mixture was agitated at 4°C for 12h and purified by dialyzes against PBS (10 mmol/L, pH 8.0) for two days. After centrifugation for 10 min, the supernatant was collected and stored at 4°C. Formation of the conjugate was confirmed with a UV-vis spectroscopy.

2.6 Production of mAb

Six-week-old female Balb/c mice were immunized at multiple sites with 50 μ g of ITeAHGA-BSA conjugate emulsified in complete Freund's adjuvant. Booster injections were given at 2-week intervals with the same amount of conjugate emulsified in incomplete Freund's adjuvant. Mice were tail bled, and the quality of the antiserum was assessed using an indirect ELISA. The mouse with the highest titer received a final intraperitoneal injection of 100 μ g of immunogen conjugate (without adjuvant) three days prior to cell fusion.

Cell fusion procedures were performed as described by Moreno et al. (2001). The spleen cells (10^8 cells) from the selected mouse were mixed with SP2/0 myeloma cells (10^7 cells) at a 10:1 ratio in 50% (w/v) PEG 2000. The fused cells were distributed in 96-well plates and cultured in HAT selection medium at 37°C in a humidified 5% CO₂

incubator.

When the hybridoma cells reached around 30-40% confluence, culture supernatants were screened for their binding activities to ITeAHGA-OVA with an indirect ELISA. The hybridomas showing the desired specificity were sub-cloned for multiple rounds by the limiting dilution method until a pure and stable antibody-producing clone was obtained. The positive clones were injected into female Balb/c mice to obtain ascitic fluid for antibody production. Antibodies in the fluid were purified by the caprylic acid-ammonium sulfate precipitation method (Zhao et al. 2002) and stored at -20°C.

2.7 Indirect Competitive ELISA (icELISA)

2.7.1 icELISA Protocol

Ninety-six well microtiter plates were coated with 100 µL/well of ITeAH-OVA overnight at room temperature. The plates were washed and incubated with 120 µL/well of blocking solution for 3 h at 37°C. After washing, 50 µL of the standard solution or sample extracts along with 50 µL of antibodies were added. Plates were incubated for 40 min and washed. Goat anti-mouse IgG-HRP was added (100 µL/well) and incubated for 30 min at 37°C. After washing, 100 µL of the chromogenic reagent was added and incubated for 10 min. The reaction was stopped by adding 50 µL of stop solution, and the absorbance was measured at 450 nm using a Plate Reader.

The results were expressed as the percentage of inhibition (B/B_0), where B and B_0 are the absorbance values of the wells with and without standard solution, respectively. The competitive standard curve was constructed by plotting the B/B_0 values against the logarithm of analyte concentration. Sigmoid curve was obtained using OriginPro 8.5 software (OriginLab Corp., Northampton, USA). The limit of detection (LOD) was determined as the 10% inhibiting concentration (IC_{10}) (Henniona and Barcelob 1998). The linear range was defined as the detection regime between the lower and

upper limits of quantification, i.e., the IC₂₀-IC₈₀ working range.

2.7.2 Optimization of Assay Conditions

The most sensitive reaction condition of the icELISA assay was achieved when using ITeAHGA-BSA, ITeAH-OVA, and ITeAH as the immunogen, coating antigen, and competition analyte, respectively. Other experimental parameters were also optimized to further improve the assay sensitivity including checkerboard titrations of coating antigens and antibody dilutions, different incubation time of antigen-antibody and secondary antibodies, as well as various buffer systems.

2.8 Cross reactivity

The specificity of the generated monoclonal antibody was assessed for its cross-reaction rate (CR) with a group of structurally similar analogues based on the IC₅₀ data calculated according to the following equation (Cui et al. 2011):

$$CR(\%) = \frac{IC_{50\text{ITeAH}}}{IC_{50\text{structural analogue}}} \times 100.$$

2.9 Sample Collection and Preparation

Twenty samples were obtained from the local supermarket, apple juice (n=5), beer (n=5), tomato ketchup (n=4), and dried fruit (n=6). The liquid samples (1mL) were extracted with 2 mL of chloroform on a wrist-action shaker for 1 min. This was repeated two times followed by centrifugation (1,000×g, 10 min). The dried fruit samples were extracted with 2:3:3 methanol-acetonitrile-water (v/v/v) for 25 min and 4:1 chloroform-ethanol (v/v) for 1 min successively at room temperature (Stinson et al. 1981). ITeA in the samples was first reduced to ITeAH using hydrazine hydrate prior to detection using the following procedures. The organic phase containing the ITeA was mixed with 100 µL hydrazine hydrate and vigorously agitated for 30 min at room temperature. The reaction was stopped by addition of 500µL of H₂O and the mixture was transferred into a 25 mL round-bottom flask where the solvent was

evaporated to dryness in a rotary evaporator at 60°C under reduced pressure. The residue was then resuspended in 1 mL H₂O. To eliminate sample matrix effects, the apple juice, beer solution and the tomato ketchup was further diluted 35-45 times with the assay buffer prior to icELISA analysis. All samples were subject to analysis by both icELISA and HPLC-MS/MS.

2.10 Recovery tests

ITeA was added to apple juice (1 mL) to give the final concentrations at 30, 150, 300 ng/mL, respectively. For the beer sample (1 mL) and tomato ketchup (1 g), the final concentrations were 150, 300, 720 ng/mL or ng/g, respectively. All of the spiked samples were prepared as described in 2.9 and measured with the developed icELISA. Calibration curve was constructed with a serial dilutions of ITeAH (0, 0.064, 0.32, 1.6, 8, 40, 200, and 1,000 ng/mL) and used to measure the concentration of ITeA from different extracted samples based on the reduction rate of 65% (ITeA to ITeAH).

2.11 HPLC-MS/MS analysis

The mobile phase was a mixture of the ammonium formate solution (5 mmol/L in water, adjusted to pH 7.8 with ammonia) (A) and acetonitrile (B), which was used in the following linear binary gradient: 0-3min, 5% B; 3-5 min, 5-15% B; 5-8 min, 15%-100% B; and 8-11 min, 100% B. The injection volume and flow rate were 50µL and 0.4 mL/min, respectively. Analytes were determined by ESI-MS/MS in the positive mode. Other parameters were as follows: gas temperature, 350°C; gas flow, 10 L/min; nebulizer gas, 50 psi; and capillary voltage, 3.5 kV.

3. Results and discussion

3.1 Hapten Synthesis and Conjugate Preparation

The design and production of functional haptens is the first and a critical step in any immunoassay development. Similar to many other small molecules, ITeA (197 Da) is

not immunogenic itself and lacks an available chemical group for protein conjugation. In this work, two novel ITeA haptens is illustrated (Fig.2). An intermediate hapten ITeAH was first prepared by condensation of hydrazine hydrate to the ketone group of ITeA. It was then reacted with glyoxalic acid to introduce the carboxyl group to obtain the tentative hapten ITeAHGA with a short aliphatic spacer arm. It has been suggested that a linear interval arm with aliphatic linkers comprised of a semi-rigid unsaturated double bond structure with three to six carbon atoms is generally good for producing the desired antibodies (Shen et al. 2007; Mercader et al. 2008). Using the same strategy, we previously reported the successful production of anti-TeA antibody and subsequently development of an ELISA for TeA (Yang et al. 2012). The successful syntheses of ITeA, ITeAH, and ITeAHGA were confirmed by MS and NMR data.

ITeA: APCI-MS, m/z 196.4 ($[M-H]^-$). 1H NMR (600 MHz, $CDCl_3$, TMS): δ 0.96 (d, $J=6.37$ Hz, 3H, CH_3), 0.98 (d, $J=6.46$ Hz, 3H, CH_3), 1.45 (m, 1H, CH), 1.84-1.67 (m, 2H, CH_2), 2.46 (s, 3H, CH_3), 3.85 (ddd, $J=9.80, 3.59, 0.88$ Hz, 1H, CH), and 6.03 (s, br, 1H, NH).

ITeAH: The APCI-MS was m/z 212.1 ($[M+H]^+$). The 1H NMR (600 MHz, $CDCl_3$, TMS): δ 0.96 (d, $J=6.43$ Hz, 3H, CH_3), 0.95 (d, $J=6.34$ Hz, 3H, CH_3), 1.33-1.41 (m, 1H), 1.72 (m, 2H), 2.67 (s, 3H, CH_3), and 3.48-4.04 (m, 1H).

ITeAHGA: APCI-MS, m/z 266.0 ($[M-H]^-$). The 1HNMR (600MHz, $DMSO-d_6$, TMS): δ 0.88 (d, $J=6.59$ Hz, 6H, $2CH_3$), 1.29 (ddd, $J=13.90, 9.47, 4.73$ Hz, 1H, H_aCH_b), 1.48 (ddd, $J=13.50, 9.31, 4.08$ Hz, 1H, H_aCH_b), 1.86-1.75 (m, 1H, CH), 2.61 (s, 3H, CH_3), 3.73 (dd, $J=9.10, 4.00$ Hz, 1H), 7.69 (s, 1H), 6.40 (s, br, 1H, NH), and 13.13 (s, br, 1H, COOH).

The production of immunogen and the homologous coating antigen was carried out by coupling the hapten ITeAHGA to the carrier protein (BSA/OVA) via the common

N-hydroxysuccinimide active ester method, while ITeAH was conjugated to OVA and used as the heterologous coating antigen through the cross-linking agent glutaraldehyde. Successful conjugations were confirmed by the UV-vis data (data not shown). The antigen was added in molar excess over that of carrier protein in order to bind to sufficiently (Hamajima et al. 1995). Most reported hapten:protein ratios are between 50:1 to 100:1 which resulted in ideal artificial antigen and subsequently produced ideal antibodies (Hamajima et al. 1995; McAdam et al. 1992; Shen et al. 2007).

3.2 mAb Production and Identification

In our experiment, the mAb raised against ITeAH was successfully produced from a selected single hybridoma (2E8) and used to establish icELISA detection system for ITeA. The derivation strategy that is based on the special antibody to the derivative of the determinants, has been successfully used by other researchers to establish immunoassays for several haptens including 1-aminohydantoin (AHD) (Jiang et al. 2012), furaltadone metabolite AMOZ (Shen et al. 2012) and TeA (Prelle et al. 2013). This strategy is particularly useful when production of antibody to toxic compound is in question, whether it is due to the extremely toxicity or the lack of toxic reagent itself. Considering the homologous coating antigens for which antibodies generally have weaker affinity towards (Xu et al. 2012; Galve et al. 2002), two coating antigens ITeAHGA-OVA and ITeAH-OVA were compared in the present study and the results (Fig. 3) clearly indicated that the heterologous coating antigen ITeAH-OVA ($IC_{50} = 14.5$ ng/mL) was superior to the homologous coating antigen ITeAHGA-OVA ($IC_{50} = 27.2$ ng/mL). This is in accordance with other research finding that the use of an appropriate heterologous coating antigen can significantly improve sensitivity of the assay (Qi et al. 2012). Subsequent experiments were therefore carried out based on the

coating antigen of ITeAH-OVA.

3.3 ELISA Optimization

To optimize the developed ELISA, we tested various concentrations of the coating antigen and antibody, reaction time of antigen with antibody, incubation time of HRP labeled secondary antibody, different analyte/antibody ratio and buffering system. The A_{\max}/IC_{50} ratio (A_{\max} is the maximum value of absorbance) from the competition curves of ITeAH was used to estimate the influence of each condition on assay performance, and the higher ratio suggests the higher sensitivity of the assay (Liang et al. 2007). Figure 4(A) shows an optimal combination of a coating antigen at concentration of 15.6 ng/mL and an antibody at 1:2,000 dilution, exhibiting the lowest IC_{50} value of 5 ng/ml. Other optimized assay conditions include 40-min reaction time (Fig 4(B)) for incubation of the antigen-antibody, 30 min for the anti-IgG-HRP antibody (Fig 4(C)), and H₂O was found as the most suitable diluent for the analyte (Fig 4(D)). Under these conditions, a calibration curve was constructed for ITeAH at concentration ranged from 0.064 to 1,000 ng/mL with a linear working range between 1.7 and 36.4 ng/mL ($R^2=0.9944$) (Fig. 5). The established icELISA system is highly sensitive with an IC_{50} value of 7.8 ng/mL and a LOD of 0.5 ng/mL for ITeAH. An enzyme immunoassay has been reported for TeA in apple and tomato products with an IC_{50} of 320 ± 130 ng/ml for TeA, but a much lower IC_{50} of 23.3 ± 7.5 ng/ml for the TeA acetate. When TeA acetate was employed as the standard in the EIA to measure the acetylated TeA, an IC_{30} of 5.4 ± 2.0 ng/ml was resulted (Gross et al. 2011). Ackermann et al.(2011) described the development of an EIA for rapid determination of alternariol, another *Alternaria* mycotoxin, with a detection limit of 1-2 µg/kg.

3.4 Cross-reactivity Studies

The specificity of the developed monoclonal antibody was examined by testing the

cross-reactivity (CR) rates of several *Alternaria* mycotoxin analogues (Table 1), including iso-tenuazonic acid (ITeA), tenuazonic acid (TeA), alternariol (AOH), alternariol methyl ether (AME), and hydrazine hydrate. The results were all less than 0.1%, suggesting the high specificity of the produced monoclonal antibody towards ITeAH, which is vital in the developed ELISA system.

3.5 Analysis of Spiked Samples

Spiked apple juice, beer, and tomato ketchup samples were analyzed using the developed ELISA. It is generally recognized that the reasonable sample preparation can effectively reduce the matrix effect (Sheng et al. 2012). A simple H₂O dilution of samples was used in this study, i.e., 35 times dilution for the extracts of apple juice and beer, and 45-fold dilution for extract of tomato ketchup. Samples were spiked with ITeA at different concentrations to evaluate the recovery rates of the developed immunoassay. As shown in Table 2, the recoveries of ITeA ranged from 93.3% to 109.8% for apple juice, 82.3% to 93.2% for beer, and 93.2% to 107.3% for tomato ketchup, respectively, and that was within the general requirement of 70-120% for screening immunoassays (Kondo et al. 2012; Wang et al. 2011). The coefficients of variation (CV) were all found to be less than 15%, indicating a good level of precision of the developed ELISA.

3.6 Comparison of the ELISA and HPLC-MS/MS Method

The ELISA results were compared and confirmed by the HPLC-MS/MS method. The linear relationship between the two methods was at $y=0.7660x +45.52$ with a squared coefficient of correlation (R^2) of 0.9557 for the spiked food samples (Fig. 6), suggesting a good agreement between the screening ELISA and confirmatory HPLC-MS/MS methods. These results also demonstrated the suitability of the developed ELISA for the detection of trace levels of ITeA in food samples. Asam et al.

(2013) reported the development of analytical methods for detection of TeA and its analogues in foods with an LOD of 1 µg/kg (TeA) and 3 µg/kg (ITeA) for derivatized samples and 60 µg/kg (TeA and ITeA) for samples without derivatization, while the ELISA method we developed here has a significantly lower LOD of 0.5 µg/L for ITeA. Taking consideration of the generally lower level of ITeA than TeA in fruits and vegetables, the present ITeA ELISA possesses lower detection limit and higher sensitivity.

3.7 Detection of ITeA in real samples

The commercial food samples (n=20) were analyzed using both ELISA and HPLC-MS/MS methods. As shown in Table 3, results revealed a highly conformity between the two methods, i.e., all samples were found to be positive by the developed ELISA and their quantified results are in consistence with the HPLC-MS/MS data. This further confirms the reliability of the established ELISA method as a fit-for-purpose screening tool for quantitative analysis of ITeA in food samples. ITeA was detected with varying concentrations in all samples tested, i.e., 39.2-110.3 ng/mL in apple juice, 45.4-79.3 ng/mL in beer, 41.5-81.1 ng/mL in tomato ketchup and 43.4-157.2 ng/mL in dried fruits. Using the UPLC-ESI-MS/MS, Walravensa et al. (2014) reported around 71% of rice samples and 31% of oat flake samples obtained in Belgium were contaminated with TeA at concentrations ranging from 1.90-113 µg/kg and 2.13-39 µg/kg, respectively. Such high frequency and levels of contamination of *Alternaria* mycotoxins and TeA in particular has highlighted the importance of continued monitoring of TeA in food and feed. Likewise, similar occurrence will also be applied to ITeA contamination scenario.

Currently, there are no details of accepted daily intake (ADI) and maximum residue limits (MRL) available for ITeA. Moreover, due to a lack of information on occurrence and toxicity, the European Food Safety Authority (EFSA) stated that a risk assessment for *Alternaria* mycotoxins in feed was not possible (EFSA, 2011). This may partially due to the lack of rapid methods such as immunoassays that are capable of screening a large number of samples within a relatively short period of time. Consequently, the liquid chromatography coupled to (tandem) mass spectrometry is the method of choice for quantification of *Alternaria* toxins in foods and feeds. The sensitive and reliable ELISA described in this study would therefore contribute greatly to the effective and efficient monitoring of ITeA in food and the environment.

4. Conclusions

In this paper, we report the development of a sensitive ELISA using a specific monoclonal antibody for reliable detection of ITeA in food samples. The optimized ELISA has an IC₅₀ value of 7.8 ng/mL and a detection limit of 0.5 ng/mL with good extraction efficiency for apple juice, beer, and tomato ketchup samples. The established immunoassay was subsequently implemented in a mini-survey of commercial food samples with results revealing a potentially widespread contamination of ITeA (probably alongside TeA) in processed commercial foods. The close agreement between the ELISA result and HPLC-MS/MS data has confirmed the reliability of this newly developed ELISA as a versatile screening tool for monitoring ITeA in different foods. Future studies will seek its applications in a wider range of foodstuffs including animal feeds to facilitate the collection of occurrence data and estimation of dietary exposure for this *Alternaria* mycotoxin.

414

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420 **Conflict of Interest:** All authors declare no conflict of interest.

421 **Ethical approval:** All procedures involving animals were approved and performed
422 in accordance with the relevant protective and administrative guidelines for laboratory
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426

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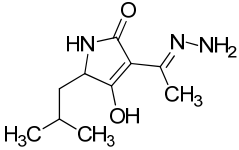
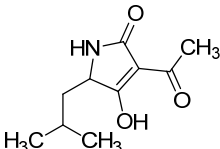
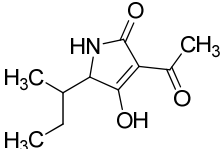
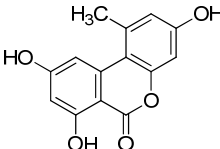
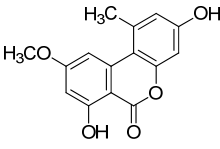
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555

556

557 **Table 1.** Cross-reactivity (CR) of the novel mAb with ITeAH and other compounds

558 using the indirect competitive ELISA developed.

559

Compound	Structure	IC ₅₀ (ng/mL)	Cross-reactivity (%)
(E)-3-(1-hydrazonoethyl)-4-hydroxy-5-isobutyl-1H-pyrrol-2(5H)-one (ITeAH)		7.4	100
Iso-tenuazonic acid (ITeA)		>8000	<0.1
tenuazonic acid (TeA)		>8000	<0.1
Alternariol (AOH)		>8000	<0.1
Alternariol methyl ether (AME)		>8000	<0.1
Hydrazine hydrate	NH ₂ -NH ₂	>8000	<0.1

560

561 **Table 2.** Recoveries of ITeA from spiked apple juice, beer, and tomato ketchup by

562 icELISA (n=3).

Matrix	ITeA spiked concentration (ng/mL or ng/g)	icELISA found (ng/mL or ng/g)	
		Recovery± SD (%)	CV (%)
apple	30	93.3 ± 10.2	10.9
juice	150	98.7±9.8	9.9
	300	109.8±10.6	9.7
beer	150	89.1±7.9	8.8
	300	93.2±8.2	8.8
	720	82.3±3.6	4.4
tomato	150	94.3±9.7	10.3
ketchup	300	93.2±11.4	12.2
	720	107.3±6.5	6.1

563

564

565 **Table 3.** Concentrations of ITeA in food samples determined by icELISA and
 566 HPLC-MS/MS.

Sample	Number	icELISA (ng/mL or ng/g)	HPLC-MS/MS (ng/mL or ng/g)	Sample	Number	icELISA (ng/mL or ng/g)	HPLC-MS/MS (ng/mL or ng/g)
apple	1	39.2±3.1	44.6±5.2	tomato	1	41.5±4.1	39.5±4.4
juice	2	98.5±9.4	103.5±8.1	ketchup	2	81.1±8.5	96.4±11.4
	3	110.3±10.2	102.8±13.9		3	28.1±2.4	39.4±3.8
	4	68.4±5.1	76.6±6.4		4	30.6±1.9	38.1±5.9
	5	82.9±7.5	79.0±8.6	dried	1	48.4±2.2	43.5±1.6
beer	1	45.4±6.7	40.8±3.9	fruit	2	83.8±10.6	92.1±6.4
	2	76.2±7.9	83.5±9.7		3	76.6±6.9	84.7±9.4
	3	69.8±4.3	80.7±7.3		4	157.2±1.3	156.9±2.7
	4	79.3±6.7	81.9±10.5		5	52.2±0.7	47.4±0.5
	5	73.5±8.9	68.4±9.5		6	43.4±1.3	44.1±0.7

567

568

569 **Figure captions**

570 **Fig. 1.** Chemical structures of TeA, ITeA and its derivatives, ITeAH and ITeAHGA.

571 **Fig. 2.** Synthesis route of hapten ITeA and the derivatives of ITeAH and ITeAHGA.

572 **Fig. 3.** Dose-dependent indirect competitive ELISA curves for ITeAH against two
573 coating antigens. The error bar represents the standard deviation of the mean (n=3).

574 **Fig. 4.** Optimization of assay conditions: (A) coating antigen concentration and
575 antibody dilution; (B) competition time of antigen-antibody; (C) IgG-HRP incubation
576 time; and (D) the diluting factor of the analyte.

577 **Fig. 5.** Calibration curve for the detection of ITeAH by icELISA. Each point
578 represents the mean results of four replicates. The vertical bars indicate the mean
579 results of the standard deviation.

580 **Fig. 6.** Comparison of icELISA and HPLC-MS/MS results for ITeA quantification.

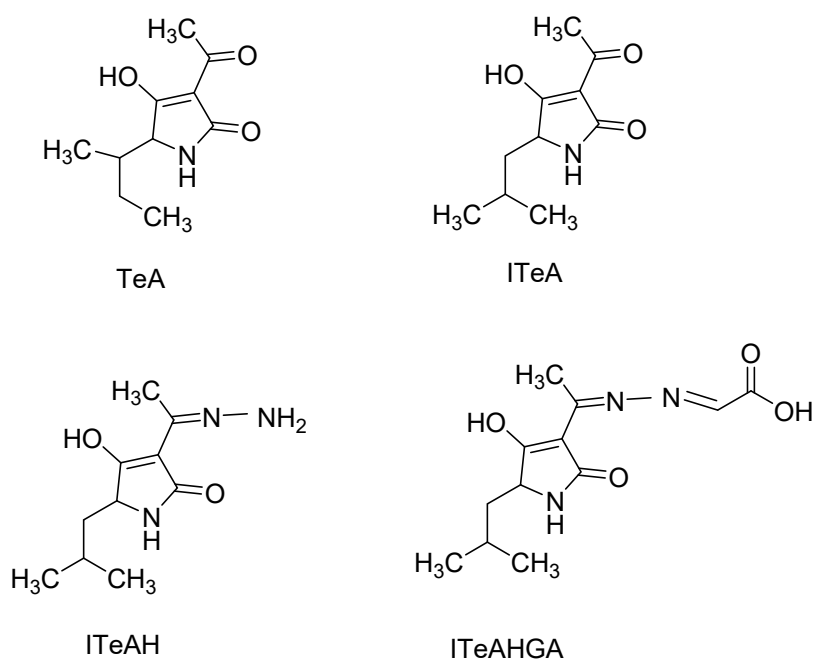


Fig. 1

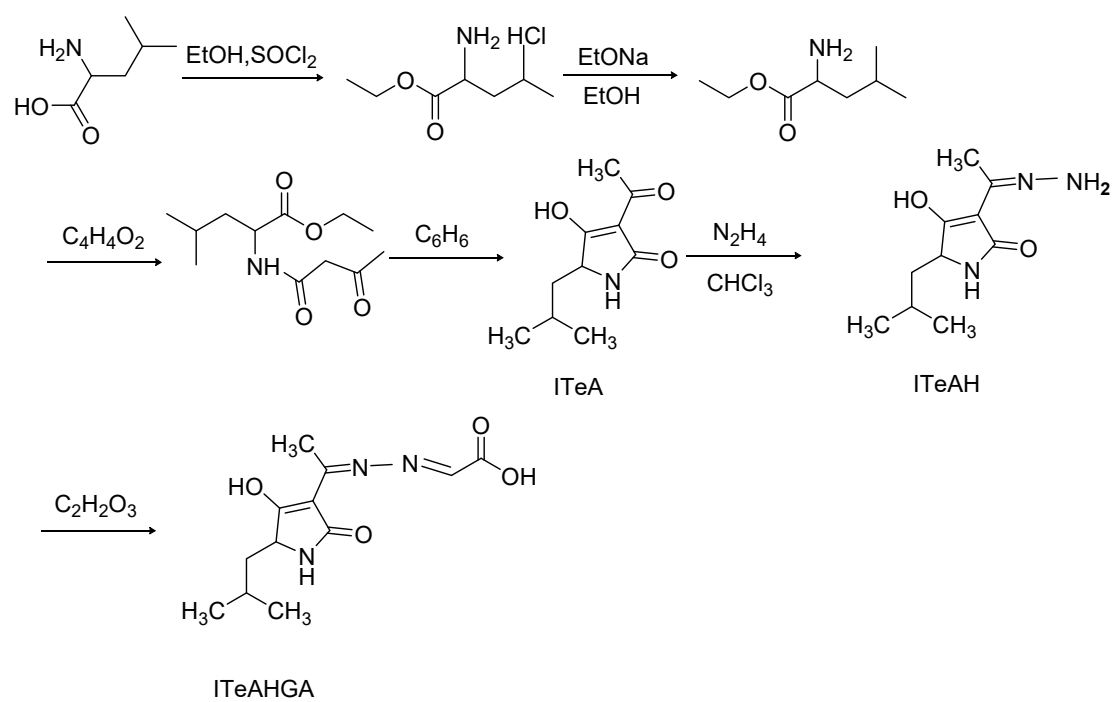


Fig. 2

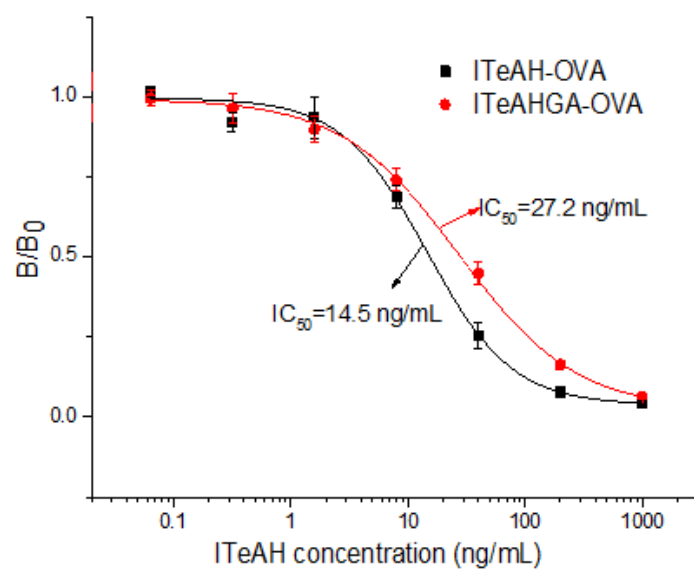


Fig. 3

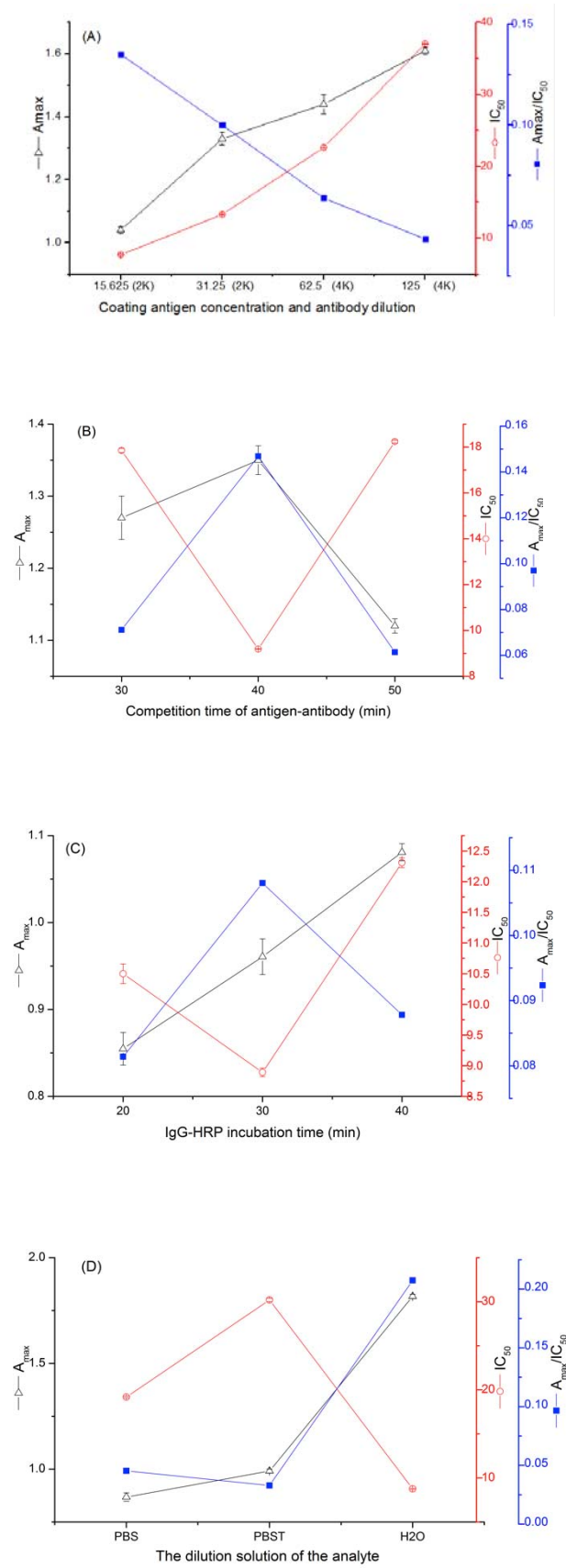


Fig. 4

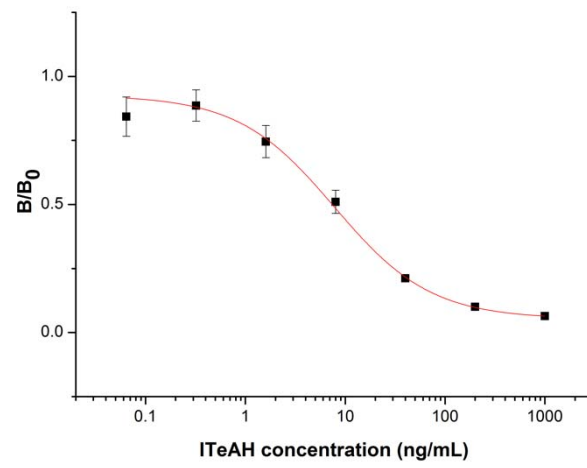


Fig. 5

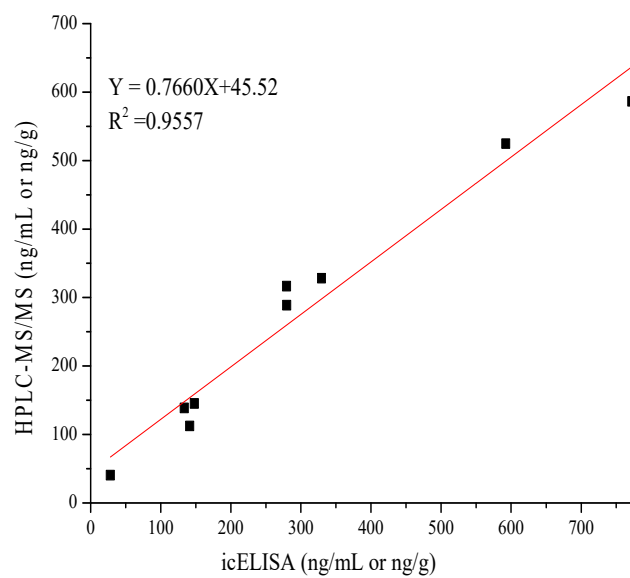


Fig. 6